

Cichoric acid production from hairy root cultures of *Echinacea purpurea* grown in a modified airlift bioreactor

Bilal Haider Abbasi,^{a,c} Rui Liu,^{a,d} Praveen K. Saxena^b and Chun-Zhao Liu^{a,d*}

Abstract

BACKGROUND: Hairy root cultures of *Echinacea* offer great potential for the production of valuable cichoric acid, but scale-up of the culture in the bioreactor represents a big challenge. Therefore, there is great interest in developing a suitable bioreactor for hairy root culture of *Echinacea* and novel bioprocessing strategies for intensifying cichoric acid production.

RESULTS: Homogenous distribution of inoculum roots and high cichoric acid production were observed in a bioreactor modified by installing a mesh draught tube with an average pore size 700 μm , slightly larger than the hairy root, about 500 μm . Improved root growth and cichoric acid production were improved by increasing the aeration rate from 0.002 $\text{m}^3 \text{h}^{-1}$ to 0.012 $\text{m}^3 \text{h}^{-1}$. The hairy root cultures in the modified bioreactor exposed once to 6 min of ultrasound treatment at day 20 gave the highest biomass accumulation of $12.8 \pm 0.3 \text{ g L}^{-1}$, which resulted in the maximum cichoric acid production of $178.2 \pm 4.9 \text{ mg L}^{-1}$ at day 30.

CONCLUSION: The present work demonstrated the effectiveness of hairy root culture in a modified airlift bioreactor. The biomass distribution remained homogenous in the modified airlift bioreactor, and the cichoric acid production was improved owing to the even root growth at optimal air flow rate. An interesting finding of this investigation was that ultrasound stimulated root growth and cichoric acid production considerably in the modified airlift bioreactor.

© 2009 Society of Chemical Industry

Keywords: airlift bioreactor; cichoric acid; *Echinacea purpurea*; hairy roots; ultrasound

INTRODUCTION

Plant cell culture has been viewed as a promising alternative to whole plant extraction for obtaining valuable chemicals.¹ At present, only a few plant cell culture processes are conducted commercially for the production of bioactive compounds. The major problems hindering the development of large-scale cultivation of plant cells include low productivity, cell line instability, and difficulty in the process of scale up. Hairy root cultures offer great potential for the production of valuable plant secondary metabolites. The advantages of using hairy roots are their independence of plant growth regulators, high growth rates, and genetic and biosynthetic stability.

Anatomical features of hairy roots including large longitudinal-radial area ratio, lateral branching and plentiful hairiness are the main reasons for entangled root matrix formation.² These structures make inoculation, tissue distribution, direct growth measurements and the harvest of roots difficult and obstruct the process of mass and energy transfer in the bioreactor culture. As a result of the factors described above, hairy roots are difficult to grow, and their scale-up in a bioreactor represents a big challenge. A number of bioreactor configurations have been examined for the growth of hairy roots, including gas-dispersed bioreactors³ and liquid-dispersed bioreactors.^{4–6} In order to achieve uniform distribution and growth of hairy roots, some internal supports, such as glass beads, polyurethane foam and stainless steel mesh, have been used.^{4,7,8} These reports have emphasized the importance of

homogeneous root distribution as a critical parameter in the root culture bioreactor design. However, high fluid flow resistance and poor oxygen mass transfer still remain a big challenge for scale-up of root culture bioreactor as hairy roots grow into a high-density root matrix.⁹

Echinacea purpurea is the most common herbal medicine due to the presence of diverse biologically active caffeic acid derivatives, especially cichoric acid. Cichoric acid has shown phagocytic, antihyaluronidase, antiviral activity and inhibited HIV-1 integrase and replication.¹⁰ Commercial production of *Echinacea* has been limited by a range of issues including contamination of plant materials by microorganisms, pollution from the environment,

* Correspondence to: Chun-Zhao Liu, National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, P.R. China. E-mail: czliu@home.ipe.ac.cn

a National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, P. R. China

b Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada N1G 2W1

c Department of Biotechnology, Quaid-i-Azam University, Islamabad-45320, Pakistan

d Graduate School of the Chinese Academy of Sciences, Beijing 100049, P. R. China

variability of active components and lack of pure, standardized plant material for biochemical analysis.¹¹ To address these issues, *Echinacea* hairy root cultures have been considered a promising source of physiologically consistent plant tissues for a more standardized production of the valuable cichoric acid.¹² The basic objective of this work was to investigate the performance of an airlift bioreactor for cultivation and cichoric acid production by hairy roots of *E. purpurea*. A concentric vertical stainless steel mesh tube was employed to provide support and facilitate distribution of the inoculated roots. In addition, the effects of pore size of the mesh draught tube and aeration rate on growth and cichoric acid biosynthesis in *E. purpurea* hairy roots were investigated. The optimized bioreactor system was used to assess the efficacy of ultrasound stimulation for enhancing root growth and cichoric acid production in *E. purpurea* hairy roots.

MATERIALS AND METHODS

Hairy root cultures

The *E. purpurea* hairy roots were initiated and maintained as described previously.¹² Fresh mass (4 g) of the hairy roots was subcultured every 21 days in 200 mL liquid MS medium¹³ with 30 g L⁻¹ sucrose in 500 mL Erlenmeyer flasks incubated at 25 ± 1 °C in the dark on a rotary shaker (Model-P270, Wuhan, China) set at 100 rpm. The medium was adjusted to pH 5.8 before autoclaving at 121 °C for 20 min.

Bioreactor system and operation

A modified airlift bioreactor for *E. purpurea* hairy roots was constructed from a flanged glass column (diameter = 10 cm, height = 25 cm) which contained a vertical stainless steel mesh cylinder (diameter = 5 cm, height = 15.5 cm) as the draft tube for attachment of the inoculum roots and an air sparger (compressed stainless steel particles) underneath the bioreactor for air supply (Fig. 1). Inside the mesh cylinder is the up-riser, and outside of the mesh cylinder is the down-comer for mixing in the airlift bioreactor. The working volume of the bioreactor, defined as the volume between the top of the mesh and the bottom of the reactor, was 1.7 L. Humidified air passed through a 0.22 µm filter (Millipore, MA) before entering the reactor through the air sparger. Before starting the culture, the bioreactor and the air supply system were sterilized by autoclaving at 121 °C for 40 min. 34 g hairy roots from 21-day-old shake flask cultures and 1.7 L sterilized MS liquid medium with 30 g L⁻¹ sucrose were added into the bioreactor system.

Stainless steel mesh with various pore sizes (380, 700, 1850, 3350 and 6700 µm) were employed to determine the performance of root growth and cichoric acid production in the modified airlift bioreactor. After a stainless steel mesh tube with suitable pore size was selected, different aeration rates (0.002, 0.004, 0.008, 0.012 and 0.016 m³ h⁻¹) were investigated and optimized for cultivating hairy roots. An ultrasonic cleaning bath (27 × 22 × 14 cm, Model KQ 5200DB, Shumei, China) with a fixed capacity of 40 kHz and variable power levels was used to insonate the hairy root cultures in the modified airlift bioreactor. The bioreactor culture was exposed to ultrasound only once for different exposure periods of 0, 2, 4, 6 and 8 min on day 20 at log growth phase and was returned to the normal culture condition afterward. For the ultrasound exposure, the bioreactors containing *E. purpurea* hairy roots were partially immersed in the sonic bath (at a power of 200 W) containing 8

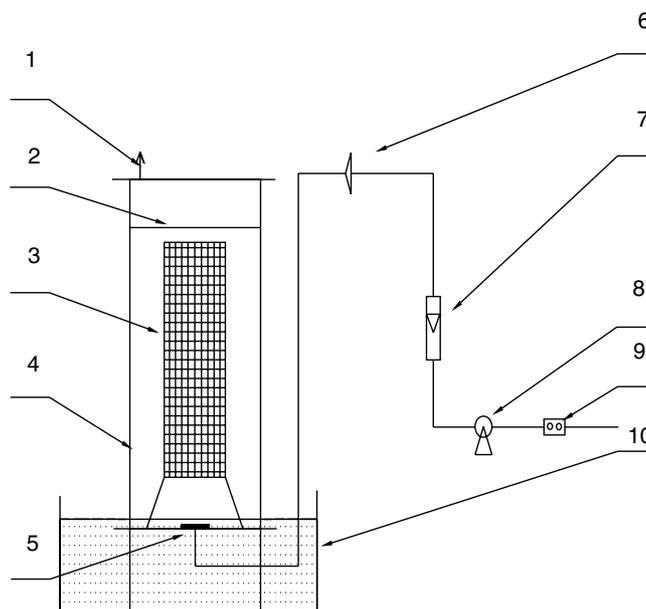


Figure 1. Diagram of a modified airlift bioreactor equipped with a vertical stainless steel mesh draught tube and placed inside ultrasonic bath. 1: air outlet; 2: level of liquid medium; 3: vertical stainless steel mesh draught tube; 4: glass column; 5: air sparger; 6: air filter; 7: airflow meter; 8: air pump; 9: electrical outlet; 10: ultrasonic bath.

L water to a depth at which the air-sparger at the bottom of the bioreactor was about 1.0 cm below the liquid in the bath. The bath temperature was maintained at 25 ± 1 °C with the built-in temperature controller and by cold water. During the ultrasound treatment, the bioreactor was left to stand vertically in water without touching the boundary wall of the ultrasonic cleaning bath (Fig. 1).

All bioreactor experiments were conducted at 25 ± 1 °C in continuous light (60 µmol m⁻² s⁻¹). There were triplicate bioreactors for each treatment, and the root cultures were harvested at day 30. Mesh draft tubes were taken out, and root beds were easily cut using a long sharp knife.

Analytical procedures

For fresh weight (FW) determination, the hairy root cultures were gently pressed on filter paper to remove excess water and weighed. Subsequently, the roots were dried in an oven at 60 °C for 24 h and dry weight (DW) was recorded. Cichoric acid was estimated according to the high performance liquid chromatography (HPLC) analytical method described by Liu *et al.*,¹² and anthocyanins and lignin were analyzed according to the methods of Harborne¹⁴ and Goering and Soest,¹⁵ respectively. Root viability was estimated by reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) method.¹⁶ The phenylalanine ammonia lyase (PAL) activity was determined according to the method described by Koukal and Conn,¹⁷ with one unit of activity (U) corresponding to an absorbance variation of 0.01.

Statistical analysis

Each of the treatments was tested three times and the data were collected after day 30. All data presented are the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Effect of pore sizes of stainless steel mesh draught tube on growth and cichoric acid production

A modified airlift bioreactor with a concentric vertical mesh draught tube was proposed for *E. purpurea* hairy root culture for the production of cichoric acid. Pore size of the mesh draught tube was found to be an important factor affecting growth and cichoric acid production of *E. purpurea* hairy roots in the modified airlift bioreactor. After inoculation, the inoculum roots (2.0 cm long) remained suspended in the liquid medium following the liquid flow pattern driven by external air supply (an aeration rate of $0.002 \text{ m}^3 \text{ h}^{-1}$ for the first 3 days, and $0.004 \text{ m}^3 \text{ h}^{-1}$ until root harvest at day 30), and became progressively trapped into the mesh draught tube (Fig. 2). Among all mesh draught tubes tested, the optimum homogeneity of inoculum roots was observed in the modified bioreactor with mesh draught tube with an average pore size of $700 \mu\text{m}$, compared with the hairy root with an average diameter of about $500 \mu\text{m}$. The hairy root cultures grew evenly along the draught tube in the modified airlift bioreactor, and completely filled the bioreactor after 30 days. As a result, the mesh draught tube with pore size $700 \mu\text{m}$ gave the highest dry weight of $7.8 \pm 0.2 \text{ g L}^{-1}$ and the maximum cichoric acid production of $102.5 \pm 5.3 \text{ mg L}^{-1}$ after 30 days (Fig. 3).

Bioreactors equipped with a vertical mesh cylinder for inoculum support were reported to be favorable to hairy root growth of *Solanum chrysotrichum*² and carrot.⁹ Our results provide the

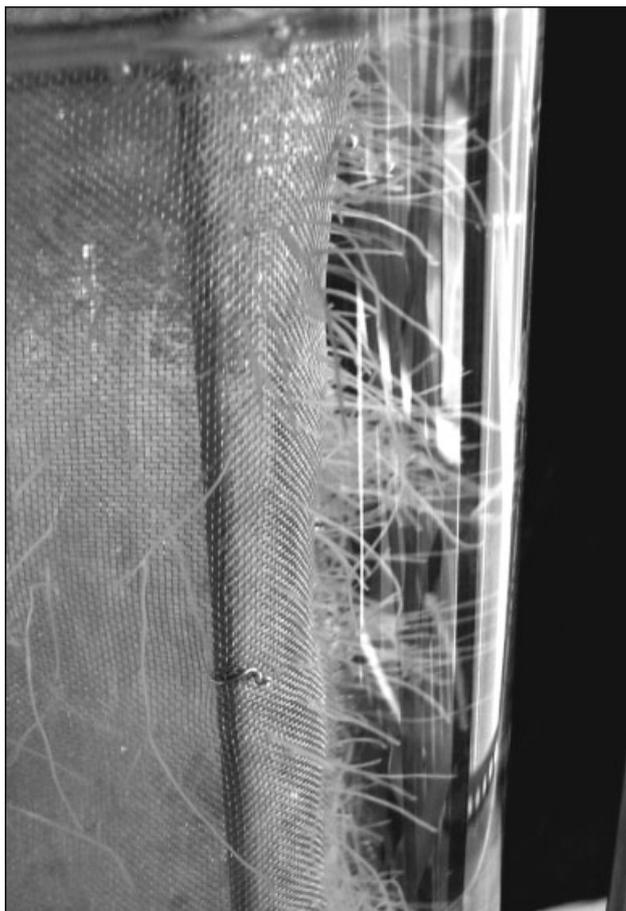


Figure 2. Homogenous attachment of *Echinacea* hairy roots to the mesh draught tube in a modified airlift bioreactor.

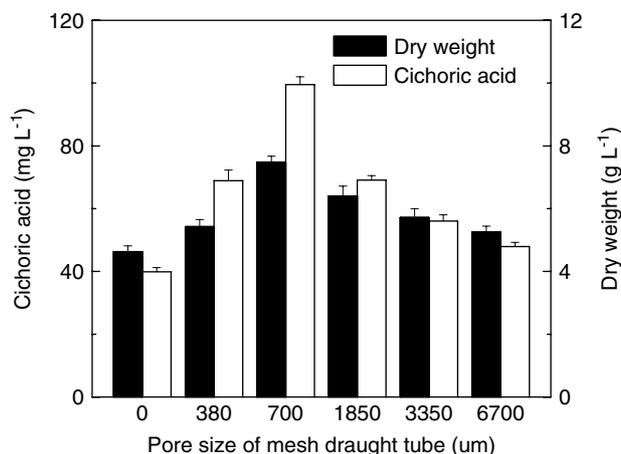


Figure 3. Effect of pore sizes of stainless steel mesh draught tube on growth and cichoric acid production of *E. purpurea* hairy roots in the modified airlift bioreactor. Values are means of triplicates with the standard deviation.

evidence that it is necessary to install a concentric mesh draught tube in the airlift bioreactor in order to avoid spatial heterogeneity developed in the culture vessel. Furthermore, it is also apparent that the pore size of the vertical mesh draught tube plays a key role in homogenous distribution of the initial root inoculum and its subsequent growth.

Effect of aeration rate on growth and cichoric acid production of *E. purpurea* hairy roots in the modified airlift bioreactor

In pneumatically agitated bioreactors, the volumetric gas flow rate is a particularly important parameter affecting the rates of oxygen transfer and broth recirculation, as well as the degree of turbulence. The modified airlift bioreactors were operated at different rates of aeration (0.002 , 0.004 , 0.008 , 0.012 and $0.016 \text{ m}^3 \text{ h}^{-1}$) after the first 3 days at an aeration rate of $0.002 \text{ m}^3 \text{ h}^{-1}$. The results presented in Fig. 4 show that the biomass increased from $5.9 \pm 0.2 \text{ g L}^{-1}$ to $9.0 \pm 0.3 \text{ g L}^{-1}$ and cichoric acid production increased from $74.3 \pm 3.2 \text{ mg L}^{-1}$ to $146.5 \pm 4.5 \text{ mg L}^{-1}$ when the aeration rate was increased from $0.002 \text{ m}^3 \text{ h}^{-1}$ to $0.012 \text{ m}^3 \text{ h}^{-1}$. No additional increments were observed in root growth or cichoric

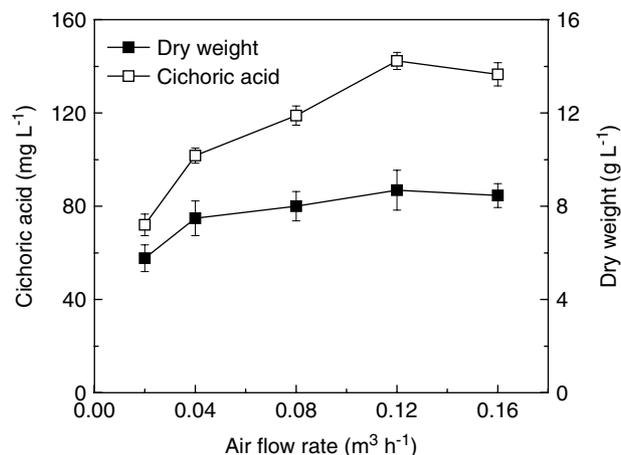


Figure 4. Effect of aeration rate on growth and cichoric acid production of *E. purpurea* hairy roots in the modified airlift bioreactor. Values are means of triplicates with the standard deviation.

acid production with aeration rate beyond $0.12 \text{ m}^3 \text{ h}^{-1}$. Specific requirements of aeration for optimum growth and biochemical output have been observed for hairy root cultures of other medicinal species.^{18,19}

Gas-liquid oxygen transfer and liquid recirculation in the airlift bioreactor increased with the increase of aeration rate in the absence of root or at a low-density of root biomass. As growth of the hairy roots proceeded, the fibrous hairy roots interlocked and formed a tangled and porous dense matrix. As a result, the fluid motion within the root bed became poor, especially at high biomass densities, which possibly resulted in some limitations of root growth and phytochemical biosynthesis due to the lack of dissolved oxygen and nutrient supply.⁵

Effect of ultrasound exposure on *E. purpurea* hairy root culture in the modified airlift bioreactor

The stimulatory effects of ultrasound exposure on secondary metabolism have been highlighted in shake flask cultures of plant suspensions.²⁰⁻²² In addition, the use of a low-energy ultrasound-assisted bioreactor showed improved biological activity through enhancing mass transfer rate of gas and liquid nutrients in microbial fermentation.²³ The application of ultrasound has the potential to enhance the growth and metabolism of *Echinacea*, but this aspect has so far remained uninvestigated for *Echinacea* hairy root growth and cichoric acid production in bioreactors.

To examine the effect of ultrasound exposure on the root growth and cichoric acid production in the modified airlift bioreactor, the *E. purpurea* hairy roots were exposed once to ultrasound on day 20 post-inoculation at a fixed ultrasound power for different time periods (0, 2, 4, 6 and 8 min). As shown in Fig. 5, all sonicated root cultures achieved higher biomass than that of control cultures without ultrasound treatment. Of various ultrasound exposure periods tested, a 6 min exposure at day 20 gave the highest biomass accumulation of $12.8 \pm 0.3 \text{ g L}^{-1}$, which resulted in the maximum cichoric acid production of $178.2 \pm 4.9 \text{ mg L}^{-1}$ at day 30

The hairy root cultures treated with 6 min of ultrasound became more purple than the control without ultrasound treatment. The onset of purple color is related to anthocyanin accumulation and has been shown to exist mainly in the flower of wild *E. purpurea* plants.²⁴ As shown in Fig. 6, a 6 min ultrasound treatment enhanced anthocyanin accumulation (Fig. 6(A)) indicated by the

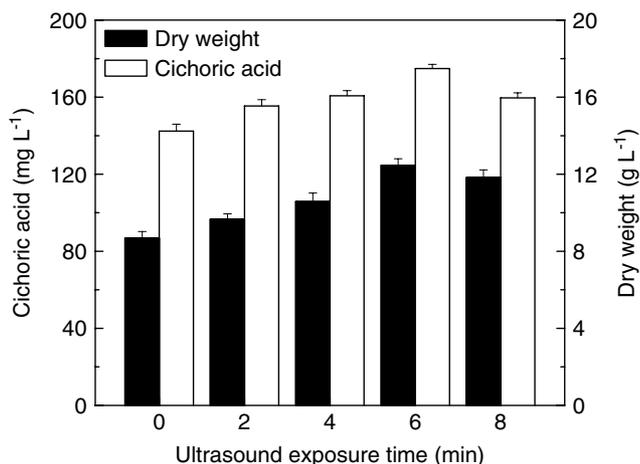


Figure 5. Effect of ultrasound exposure on growth and cichoric acid production of *E. purpurea* hairy roots in the modified airlift bioreactor. Values are means of triplicates with the standard deviation.

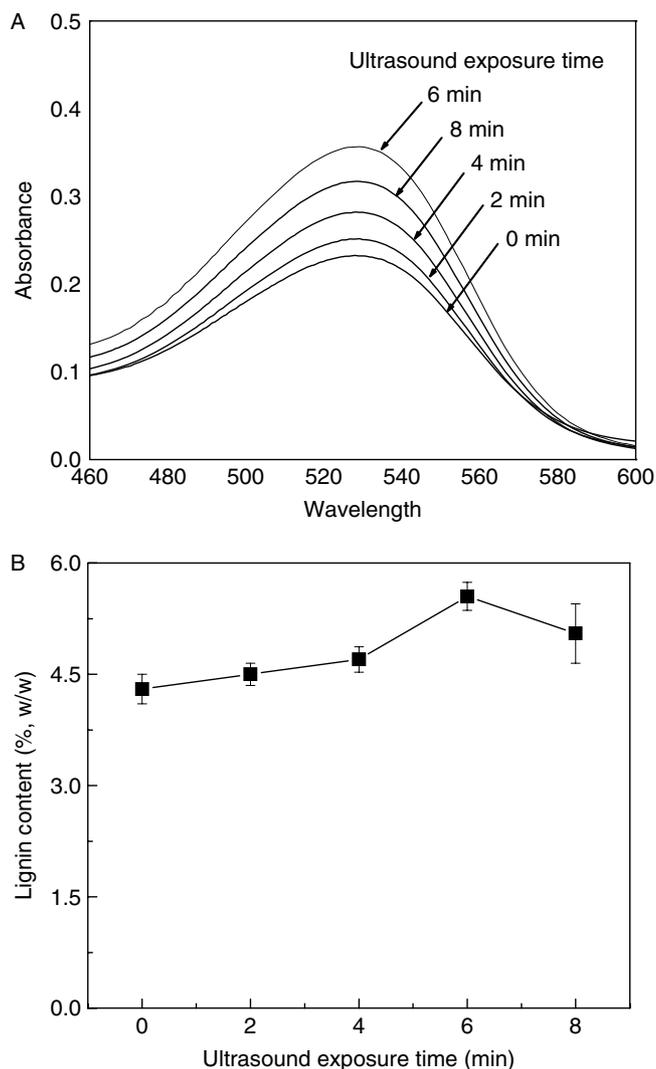


Figure 6. Effect of ultrasound exposure on anthocyanin (A) and lignin (B) accumulation in *E. purpurea* hairy roots in the modified airlift bioreactor. Values are means of triplicates with the standard deviation.

wavelength maximum between 510 and 540 nm²⁵ and lignin content (Fig. 6(B)) in the hairy root cultures, which protected the plant tissue from the physical stress. As shown in Fig. 7, the increased accumulation of anthocyanins and lignin correlated well with the increase of ultrasound-stimulated activity of PAL, a key enzyme linked to the biosynthesis of anthocyanins, caffeic acid derivatives and lignin in plant cells.²⁶ Exposure to ultrasound for more than 6 min resulted in destruction of the root cells. As a result, accumulation of cichoric acid, anthocyanin and lignin declined in the hairy root cultures.

During the ultrasound treatment, many small air bubbles released from the dense root bed were observed in the modified airlift bioreactor, along with an increase of root cell viability after the ultrasound treatment (Fig. 7). The pulsation of microbubbles of gas in the fluid generates microstreaming and other effects that might thin the fluid boundary layer around hairy roots positioned close to the bubbles, thus enhancing mass transfer of oxygen and nutrient transfer from the liquid medium to the hairy roots and removal of carbon dioxide within the high-density root matrix.²⁷ Suitably controlled ultrasonication has shown beneficial effects in

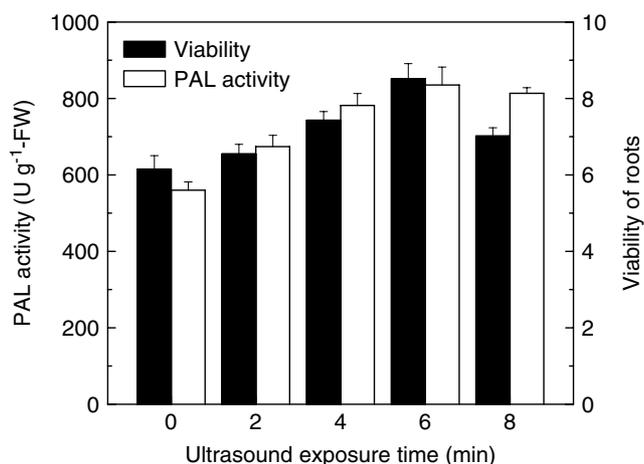


Figure 7. Effect of ultrasound exposure on PAL activity and viability of *E. purpurea* hairy roots in the modified airlift bioreactor. Values are means of triplicates with the standard deviation.

biological systems and biotechnological processes. These effects appear to have multiple mechanisms that remain to be clarified.

CONCLUSIONS

The present work demonstrated the effectiveness for hairy root culture of a modified airlift bioreactor equipped with a vertical mesh inoculum support, which has a suitable pore size for hairy root entrapment. The biomass distribution remained homogenous in the modified airlift bioreactor, and cichoric acid production was improved due to the even root growth at an optimal air flow rate. The interesting finding of this investigation was that ultrasound stimulated root growth and cichoric acid production in the modified airlift bioreactor. Together, these results present new opportunities and challenges for understanding the mechanisms of multiple function of ultrasound on biological systems and associated biotechnological processes involved in developing novel bioprocessing strategies and the sonobioreactors.

ACKNOWLEDGEMENT

This work is financially supported by National Basic Research Program (973 Program) of China (No.2007CB714301).

REFERENCES

- Liu CZ and Cheng XY, Enhancement of phenylethanoid glycosides biosynthesis in cell cultures of *Cistanche deserticola* by osmotic stress. *Plant Cell Rep* **27**:357–362 (2008).
- Caspeta L, Nieto I, Zamilpa A, Alvarez L, Quintero R and Villarreal LL, *Solanum chrysotrichum* hairy root cultures: characterization, scale-up and production of five antifungal saponins for human use. *Planta Med* **71**:1084–1087 (2005).
- Hilton MG and Rhodes MJC, Growth and hyoscyamine production from hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. *Appl Microbiol Biotechnol* **33**:132–138 (1990).
- Liu CZ, Wang YC, Zhao B, Guo C, Ouyang F and Ye HC et al, Development of a nutrient mist bioreactor for growth of hairy roots. *In Vitro Cell Dev Biol Plant* **35**:271–274 (1999).
- Liu CZ, Towler MJ, Medrano G, Cramer CL and Weathers PJ, Production of mouse interleukin-12 is greater in tobacco hairy roots grown in a mist reactor than in an airlift reactor. *Biotechnol Bioeng* **102**:1074–1086 (2009).
- Wyslouzil BE, Waterbury RG and Weathers PJ, The growth of single roots of *Artemisia annua* in nutrient mist reactors. *Biotechnol Bioeng* **70**:143–150 (2000).
- Taya M, Yoyoma A, Kondo O, Kobayashi T and Matsui C, Growth characteristics of plant hairy roots and their cultures in bioreactors. *J Chem Eng Japan* **22**:84–89 (1989).
- Whitney PJ, Novel bioreactors for the growth of roots transformed by *Agrobacterium rhizogenes*. *Enzyme Microb Technol* **14**:13–17 (1992).
- Kondo S, Honda H, Taya M and Kobayashi T, Comparison of growth properties of carrot hairy root in various bioreactors. *Appl Microbiol Biotechnol* **32**:291–294 (1989).
- Lin Z, Neamati N, Zhao H, Kiryu Y, Turpin JA and Aberham C, et al, Cichoric acid analogues as HIV-1 integrase inhibitors. *J Med Chem* **42**:1401–1414 (1999).
- Abbasi BH, Saxena PK, Murch SJ and Liu CZ, Echinacea biotechnology: challenges and opportunities. *In Vitro Cell Dev Biol-Plant* **43**:481–492 (2007).
- Liu CZ, Abbasi BH, Min G, Murch SJ and Saxena PK, Caffeic acid derivatives production by hairy root cultures of *Echinacea purpurea*. *J Agric Food Chem* **54**:8456–8460 (2006).
- Murashige T and Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**:473–497 (1962).
- Harborne JB, Spectral methods of characterizing anthocyanins. *Biochem J* **70**:22–28 (1958).
- Goering HK and Soest PJ, in *Agricultural handbook No. 379. Forage Fiber Analyses: Apparatus, Reagents, Procedures and some Applications*. Washington, USA. Pp. 20–36 (1970).
- Steponkus PL and Lanphear FO, Refinement of the triphenyl tetrazolium chloride method of determining cold injury. *Plant Physiol* **42**:1423–1426 (1967).
- Koukol J and Conn E, The metabolism of aromatic and properties of the phenylalanine deaminase of *Hordeum vulgare*. *J Biol Chem* **236**:2692–2698 (1961).
- Jeong GT and Park DH, Comparative evaluation of modified bioreactors for enhancement of growth and secondary metabolite biosynthesis using *Panax ginseng* hairy roots. *Biotechnol Bioprocess Eng* **10**:528–534 (2005).
- Liu C, Wang Y, Guo C, Ouyang F, Ye H and Li G, Enhanced production of artemisinin by *Artemisia annua* L hairy root cultures in a modified inner-loop airlift bioreactor. *Bioprocess Eng* **19**:389–392 (1998).
- Lin L and Wu J, Enhancement of shikonin production in single- and two-phase suspension cultures of *Lithospermum erythrorhizon* cells using low-energy ultrasound. *Biotechnol Bioeng* **78**:81–88 (2002).
- Wu J and Ge X, Oxidative Burst, Jasmonic acid biosynthesis, and taxol production induced by low energy ultrasound in *Taxus chinensis* cell suspension cultures. *Biotechnol Bioeng* **85**:714–721 (2004).
- Wu J and Lin L, Ultrasound-induced stress responses of *Panax ginseng* cells: enzymatic browning and phenolics production. *Biotechnol Prog* **18**:862–866 (2002).
- Schlafer O, Sievers M, Klotzbucher H and Onyeché TI, Improvement of biological activity by low energy ultrasound assisted bioreactors. *Ultrasonics* **38**:711–716 (2000).
- Cheminaat A, Brouillard R, Guerne P, Bergmann P and Rether B, Cyanidin 3-malonylglucoside in two *Echinacea* species. *Phytochem* **28**:3246–3247 (1989).
- Goiffon JP, Mouly PP and Gaydou EM, Anthocyanic pigment determination in red fruit juice, concentrated juices and syrups using liquid chromatography. *Anal Chim Acta* **382**:39–50 (1999).
- Abbasi BH, Tian CL, Murch SJ, Saxena PK and Liu CZ, Light-enhanced caffeic acid derivatives biosynthesis in hairy root cultures of *Echinacea purpurea*. *Plant Cell Rep* **26**:1367–1372 (2007).
- Chisti Y, Sonobioreactors: using ultrasound for enhanced microbial productivity. *Trends Biotechnol* **21**:89–93 (2003).